Journal of Biochemistry and Molecular Biology, Vol. 32, No. 1, January 1999, pp. 6–11

JOURNAL OF
Biochemistry
and
Molecular Biology

Characterization of Phospholipid and Fatty Acid Composition in the Amp 1-4 Mutant Compared to Wild-Type *Arabidopsis thaliana*

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Received 31 August 1998, Accepted 23 September 1998

To understand the function of phospholipids and their fatty acid composition on the morphological changes in the amp 1-4 mutant of *Arabidopsis*, the mutant was compared to the wild-type *Arabidopsis* by TLC, HPTLC, phosphorous assay, HPLC, and GC. In the mutant, phosphatidylethanolamine (PE) was increased 5-fold and phosphatidylglycerol (PG) was decreased 1.2-fold (nmol phosphorous/g tissue). Inositol phospholipids showed a generally increased trend ranging from 1.4- to 3.0-fold (nmol inositol/g tissue). When fatty acid composition of the mutant was compared to the wild-type, linoleic (18:2) and linolenic (18:3) acids of phosphatidylcholine (PC) and PG were decreased but palmitoleic acid (16:1) and oleic acid (18:1) of PC was increased 2.5- and 2.1-fold (mol%), respectively. In galactolipids, myristic acid (14:0) of monogalactosyldiacylglycerol (MGDG) were increased 5.8-fold (mol%). Among the inositol phospholipids, lysophosphatidylinositol (L-PI) and phosphatidylinositol 4,5-bisphosphate (PIP_2) showed 4- and 1.9-fold (mol%) increase of 16:1, respectively. These results suggest that the increase of PE, the decrease of PG, the increase of inositol phospholipids, and the altered fatty acid composition are related to the phenotypic changes affecting the morphological features, and might cause different physiological changes in the amp 1-4 mutant compared to wild-type *Arabidopsis*.

Keywords: Amp 1-4 *Arabidopsis* mutant, Fatty acid, Phospholipid.

Introduction

Altered meristem program (amp) 1, a mutant of *Arabidopsis thaliana*, has high cytokinin levels and altered phenotypes such as altered embryonic pattern, faster vegetative growth, constitutive photomorphogenesis and precocious flowering (Chaudhury *et al.*, 1993). In the course of our investigation to understand plant development, we screened developmental mutants from M2 population of γ-ray irradiated *Arabidopsis* seeds. From the screening, a mutant was isolated with various morphological abnormalities. Upon characterization of the mutant phenotypically, similar phenotypes to the amp 1 mutant (Chaudhury *et al.*, 1993) was observed in several aspects of developmental changes. Our complementation test revealed that the mutant was allelic to amp 1. Thus, the mutant was named as the amp 1-4 mutant. However, amp 1-4, the new allele of amp 1, had additional phenotypic alterations such as morphological abnormalities in cotyledons, apical meristem, rosette leaves, stems, excess of root masses, transparent bubble-like structures on the apical meristem, leaves, and stems with more severe phenotypic changes compared to amp 1.

In the present study, we investigated the lipid composition of amp 1-4 mutant compared to wild-type *Arabidopsis* to determine if the phenotypic and genotypic differences in the mutant are associated with differences in the lipid composition. The objectives of our comparative study were (a) to characterize the alterations in the phospholipid and fatty acid composition and (b) to understand cellular and molecular basis of the morphological changes in the amp 1-4 mutant.

Materials and Methods

Plant materials and growth conditions For the isolation of morphological mutants, γ-ray mutagenized M2 seeds of *A. thaliana*, ecotype NO, were obtained from F. Ausubel (Massachusetts General Hospital, Boston, USA) and 10,000 seeds

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were planted on soil at the density of 1000 seeds per flat (60 cm × 30 cm) for mutant screening. Plants were grown in flats in a green house under standard conditions of 70% relative humidity and 16/8 h of light/dark cycle, and screened for amp 1-4 mutants. For the experiments, the amp 1-4 mutants were grown on 0.8% agar plate in a culture room at 28°C for 3–4 wk in parallel with wild-type *A. thaliana*, ecotype NO.

Complementation of amp 1-4 To determine allelism between amp 1-4 and amp1 (Chaudhury *et al.*, 1993), amp 1-4 was crossed to amp 1 plants and the phenotype of F1 and F2 progenies of the cross were scored.

Lipid extraction, separation, and quantification Powdered frozen whole plants were extracted for total lipids and separated by thin layer chromatography (TLC) following the procedures of Lee *et al.* (1996a; 1996b). Alternatively, inositol phospholipids were extracted using $CHCl_3$/ CH_3OH/ 12 N HCl (200:100:1.5, v/v/v) (Augert *et al.*, 1989). Individual phospholipids, inositol phospholipids, and galactolipids were separated from the total lipid extracts by two sequential TLCs on precoated silica gel plates (Kieselgel 60, Merck), using $CHCl_3$/ CH_3COCH_3/ CH_3OH/ CH_3COOH/ H_2O (40:15:18:12:10, v/v/v/v/v) for the first TLC and $CHCl_3$/ CH_3OH/ NH_4OH/ H_2O (86:76:6:16, v/v/v/v) for the second HPTLC, respectively (Pak and Larner, 1992; Pak *et al.*, 1992; 1993; 1998). After the TLC separation, individual lipid spots were identified under UV light after spraying with primulin by comparison with authentic standards. Individual phospholipid bands were scraped from TLC plates and the phosphorous content was quantified by the method of Duck-Chong (1979).

Identification and quantification of inositol phospholipids by HPLC To verify the presence of inositol and to quantify inositol from the inositol phospholipids isolated from two sequential TLCs, individual inositol phospholipid bands from the TLC plates were analyzed by Dionex anion exchange HPLC (Pak *et al.*, 1992; 1993; 1998). Briefly, individual inositol phospholipid bands isolated from two sequential TLCs were eluted from the TLC plates and hydrolyzed with 6 N HCl at 110°C for 48 h. Hydrolysates were lyophilized, dissolved in H_2O, and microcentrifuged through nylon membranes (0.2 μm, Costar Spin-X 8169, Cambridge, USA). Samples were then dried in a SpeedVac, resuspended in 25 μl of H_2O, and analyzed by a Dionex CarboPac MA-1 column with MA-1 guard column on a Dionex DX-300 in a LCM-3 Gradient System using the isocreatic system of 80 mM NaOH. All samples were analyzed at a flow rate of 0.4 ml/min and a running time of 25 min by a Pulsed Electrochemical Detector as previously described (Pak *et al.*, 1992; 1993; 1998). Inositol was identified and quantified by comparing the HPLC profile of authentic myo-inositol standard.

Fatty acid analysis by GC The individual lipid samples isolated from two sequential TLCs were subjected to methanolysis by incubating in 5% HCl-CH_3OH solution at 80°C for 2 h. The transmethylated fatty acid methyl esters (FAME) were extracted in hexane and analyzed by GC using a 30 m × 0.25 mm SP-2330 fused silica capillary column (Supelco Inc., Bellefonte, USA). The oven temperature was maintained at 170°C and the head pressure of the carrier gas (N_2 and He) was 60 psi; a split injection was used. Injector and detector temperatures were maintained at 230°C and 250°C, respectively (Lynch and Thompson, 1984).

Results

Isolation and complementation of amp 1-4 In order to study plant development, we initiated a screening of 10,000 M2 seedlings of γ-ray mutagenized seeds for developmental mutants of *Arabidopsis*. The screening resulted in the isolation of a developmental mutant whose phenotype was caused by a single recessive mutation that mapped to chromosome 3. Recently, Chaudhury *et al.*, (1993) reported an *Arabidopsis* mutant, amp 1, which had a similar phenotype as amp 1-4 and mapped to nearly the same position on chromosome 3. Thus, we did a complementation test between the two mutants to find out whether amp 1-4 was allelic to amp 1. The F1 progeny of the cross amp 1 × amp 1-4 had an identical phenotype as amp 1, indicating that amp 1-4 is allelic to amp 1 (Hwang, I., unpublished data).

Morphological changes during the developmental stage of amp 1-4 mutant It has been shown that mutation in the amp 1 gene of *Arabidopsis* has pleiotropic effects on plant development with six times more endogenous cytokinin than the wild-type (Chaudhury *et al.*, 1993). As in the case of other amp 1 mutant alleles, the amp 1-4 mutant showed various phenotypic changes in comparison to the wild-type, such as variable numbers of cotyledons, short petioles, and excess number of rosette leaves during the developmental stage. The mutant also showed much smaller siliques with small size seeds and had grossly enlarged apical meristem with slightly yellowish leaves. Interestingly, when we measured the growth rate of roots between the amp 1-4 and wild-type following various time points, the growth rate of the root was two times slower in amp 1-4 than in the wild-type. Further, the mutant had multiple roots and greatly overamplified lateral roots resulting in an excess of root mass on MS plates, whereas wild-type had a single root.

Changes in phospholipid and inositol phospholipid composition of amp 1-4 mutant As described in Materials and Methods, lipids were extracted from 3-4-wk old whole plants, separated into individual phospholipid classes and quantified by phosphorous assay. Table 1 shows that there were significant changes in the levels of phospholipid classes between the wild-type and amp 1-4 mutant of *Arabidopsis*. In the mutant, PE was increased 5-fold (wild: 5.8, mutant: 29.1 nmol phosphorous/g tissue) and PA was slightly increased (wild: 139.5, mutant: 155.0 nmol phosphorous/g tissue). In contrast, PG was decreased 1.2-fold (wild: 283.2, mutant: 230.2 nmol phosphorous/g tissue) and PC was slightly decreased (wild: 152.1, mutant: 145.0 nmol phosphorous/ gm tissue).

Table 1. Changes in phospholipid composition (nmol phosphorous/g tissue) of whole plants between the amp 1-4 mutant and wild-type *Arabidopsis thaliana*, ecotype NO.

	Wild-type	Mutant
Phospholipid	nmol phosphorous/g tissue	
PE	5.8 ± 2.6	29.1 ± 5.8
PA	139.5 ± 5.8	155.0 ± 5.2
PG	283.2 ± 10.3	230.2 ± 6.5
PC	152.1 ± 3.2	145.0 ± 6.8
Total	580.6	559.3

The results are the averages ± SD from three independent experiments.

Table 2. Changes in inositol phospholipid composition (nmol inositol/g tissue) of whole plants between the amp 1-4 mutant and wild-type *Arabidopsis thaliana*, ecotype NO.

	Wild-type	Mutant
Inositol Phospholipid	nmol inositol/g tissue	
PI	26.1 ± 0.6	39.2 ± 0.8
L-PI	7.5 ± 0.4	22.1 ± 0.3
PIP	4.1 ± 0.2	5.8 ± 0.03
PIP_2	3.2 ± 0.4	5.2 ± 0.3
Total	40.9	72.3

The results are the averages ± SD from three independent experiments.

Individual inositol phospholipids separated from two sequential TLCs were verified for the presence of inositol and quantified for inositol by Dionex anion exchange HPLC analysis as described in Materials and Methods. Table 2 compares the profiles of changes in the levels of inositol phospholipid classes between the wild-type and amp 1-4 mutant of *Arabidopsis*. In the amp 1-4 mutant, changes in the relative levels of all the inositol phospholipid classes showed an increased trend compared to wild-type. PI was increased 1.5-fold (wild: 26.1, mutant: 39.2 nmol inositol/g tissue), L-PI 3.0-fold (wild: 7.5, mutant: 22.1 nmol inositol/g tissue), PIP 1.4-fold (wild: 4.1, mutant: 5.8 nmol inositol/g tissue), and PIP_2 1.6-fold (wild: 3.2, mutant: 5.2 nmol inositol/g tissue), respectively.

Changes in fatty acid composition of amp 1-4 mutant

When the results of fatty acid analysis of individual phospholipids and galactolipids were compared between amp 1-4 mutant and wild-type, as shown in Table 3, PC and PG of the mutant revealed that polyunsaturated fatty acids, 18:2 and 18:3, were decreased but 14:0, 16:1 (2.5-fold, mol%), 18:0, and 18:1 (2.1-fold, mol%) were increased in PC, and 14:0, 16:0, and 18:1 were increased relatively less in PG. PA and PE of the mutant showed no significant changes in their fatty acids compared to wild-type. In galactolipids, MGDG and DGDG, all the fatty acids, except for a slight decrease of 18:3, were increased upto 5.8-fold (mol%) in 14:0 of MGDG compared to wild-type.

Table 4 shows the relative changes in the fatty acid composition of inositol phospholipids between amp 1-4 mutant and wild-type. PI did not show significant changes, except for a slight decrease of 18:3 in the mutant. L-PI showed decreases of 16:0 and 18:3, and 4-fold (mol%) increase of 16:1. In the case of PIP, all the fatty acids showed a decreased pattern except slight increases of 18:1 and 18:2. PIP_2 did not show any significant changes but 16:1 was increased 1.9-fold (mol%) and 18:3 decreased 1.9-fold (mol%).

Discussion

This comparative study was conducted to examine the alterations of lipid composition (mainly, phospholipids, galactolipids, and inositol phospholipids), and their fatty acids of amp 1-4 mutant compared to wild-type *A. thaliana* (ecotype NO), to examine if the mutant's various morphological abnormalities including phenotypic alterations in cotyledons, apical meristem, rosette leaves, stems, excess of root masses, and transparent bubble-like structures on the apical meristem, leaves, and stems, are associated with differences in the lipid composition, and further to correlate the lipid alteration as the cellular and molecular basis of the morphological changes in the amp 1-4 mutant.

Although it is difficult to establish whether alterations in lipid and fatty acid composition are causally and directly related to the morphological changes, our approach evolved from the notion that increase or decrease in fatty acid unsaturation and regulation of phospholipids would alter membrane fluidity and stabilization of membrane structure and function. Thus, it is more appropriate to consider how membrane destabilization resulting from alterations in the lipid composition influence the aforementioned severe phenotypic changes of the amp 1-4 mutant with respect to the cellular and molecular morphology of cellular membranes.

A correlation was found between changes in physical properties of cellular membrane and alterations in the pattern of phospholipids with combinations of fatty acids (Dicken and Thompson, 1982). Such observations suggest that stability of cellular membrane may be strongly influenced by structural parameters of the lipids, namely phospholipid and fatty acid composition (Patterson *et al.*, 1978; Raison *et al.*, 1982). Table 1 shows that there were significant changes in the levels of phospholipid classes between the wild-type and amp 1-4 mutant of *Arabidopsis*. In the mutant, PE was increased 5-fold and PA was slightly increased. In contrast, PG was decreased 1.2-fold and PC was slightly decreased. When the results of fatty acid

Table 3. Changes of fatty acid composition (mol% of total fatty acids) in phospholipids and galactolipids of whole plants between the amp 1-4 mutant and wild-type *Arabidopsis thaliana*, ecotype NO.

	PC		PG		PA		PE		MGDG		DGDG	
	[a]W	M	W	M	W	M	W	M	W	M	W	M
	Fatty Acids (mol %)											
[b]14:0	1.7	3.0	3.2	4.1	2.0	1.7	6.3	6.0	0.2	1.4	1.3	2.2
16:0	25.2	22.7	23.6	26.1	20.5	18.9	25.8	25.0	3.5	5.9	19.9	20.0
16:1	2.7	6.7	12.3	11.9	3.6	3.5	8.5	8.9	1.8	4.4	3.1	4.7
18:0	4.6	5.6	6.5	6.3	5.2	4.7	10.1	8.5	0.6	1.3	2.6	2.7
18:1	7.3	15.2	12.8	16.9	7.2	8.6	15.4	12.6	[c]_	_	2.9	5.6
18:2	29.6	24.3	23.4	19.9	35.5	38.3	19.2	23.9	5.9	9.0	7.5	11.0
18:3	28.9	22.4	18.2	14.9	26.0	24.4	14.7	15.0	87.9	78.1	62.6	54.0

The results are the averages obtained from three independent experiments.
[a]W: wild-type; M: amp 1-4 mutant.
[b]14:0, myristic acid; 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, α-linolenic acid. The number preceding the colon represents the number of carbon atoms in the fatty acid and the number following the colon indicates the number of double bonds present.
[c]_, not detected.

Table 4. Changes of fatty acid composition (mol% of total fatty acids) in inositol phospholipids of whole plants between the amp 1-4 mutant and wild-type *Arabidopsis thaliana*, ecotype NO.

	PI		L-PI		PIP		PIP_2	
	Wild-type	Mutant	Wild-type	Mutant	Wild-type	Mutant	Wild-type	Mutant
	Fatty Acids, mol %							
[a]16:0	18.5	22.4	19.9	15.7	27.1	20.3	23.0	20.6
16:1	2.7	2.0	[b]_	3.9	12.2	10.4	12.3	23.6
18:0	4.1	4.0	4.5	4.6	10.5	8.8	9.0	6.6
18:1	6.4	7.0	7.3	8.8	17.2	21.6	19.7	17.9
18:2	38.5	39.5	27.5	30.8	27.0	33.9	32.9	29.7
18:3	29.9	25.0	40.8	36.2	5.9	4.9	3.1	1.6

The results are the averages obtained from three independent experiments.
[a]16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, α-linolenic acid. The number preceding the colon represents the number of carbon atoms in the fatty acid and the number following the colon indicates the number of double bonds present.
[b]_, not detected.

analysis of individual phospholipids were compared between amp 1-4 mutant and wild-type, PC and PG of the mutant revealed that polyunsaturated fatty acids, 18:2 and 18:3, were decreased but saturated and monounsaturated fatty acids, 14:0, 16:1 (2.5-fold), 18:0, and 18:1 (2.1-fold) were increased in PC, and 14:0, 16:0, and 18:1 of PG were relatively less increased (Table 3). Thus, our findings reveal not only the sharp difference in the fatty acid composition but also the dramatic difference in the metabolic activities which regulate or influence lipid composition and membrane function.

Our study demonstrates that there are significant differences in the phospholipid and fatty acid compositions between the amp 1-4 mutant and wild-type *Arabidopsis*. The difference was especially evident when the phospholipids of the amp 1-4 mutant was compared to the wild-type. As shown in Table 1, in particular, PE was dramatically increased by 5-fold in the mutant. Although it can not be explained how the increase of PE is causally related to all the phenotypic changes of the mutant, the increase of PE, nonbilayer-forming lipids may result in formation of the lamellar-to-hexagonal phase (H_{II}) transition in the course of the plant development, which decreases the surface area of the hydrophilic (headgroup)

region resulting in greater lateral pressures in the interior of the lipid monolayer than in the surface region (Uemura and Steponkus, 1994). These packing stresses result in a spontaneous bending or intrinsic curvature in the monolayer of cellular membrane (Gruner, 1989; Tate *et al.*, 1991). It has been suggested that in the process of membrane fusion, 'lipidic particles' consisting of PE are formed in a state intermediate between a bilayer and H_{II} phase, which in turn forms an inverted micelle sandwiched between the leaflets of the membrane at the site of fusion (Rand, 1981; Rand and Parsegian, 1984). Thus, the increase of PE might cause possible endocytotic vesiculation (Dowgert and Steponkus, 1984) or/and exocytotic extrusion (Gordon-Kamm and Steponkus, 1984) of the cellular membrane of the mutant, evidenced by the morphological changes such as transparent bubble-like structures on the apical meristem, leaves, and stem, over-proliferation of the apical meristem, expansion and disorganization of cells in leaves and cotyledons, multiple or fused stems, and the abnormal spiral pattern of the leaf and flower.

PG, MGDG, and DGDG are exclusively localized in chloroplast, especially on the thylakoid membrane, and associated closely with the photosystem. PG in the thylakoid membrane is preferentially involved in protein-lipid interactions (Maroc *et al.*, 1987; Li *et al.*, 1990). Thus, it is quite probable that changes in the extent of unsaturation of fatty acids in PG, MGDG, and DGDG can modify the molecular environments of the photosystem. In fact, the degree of unsaturation of the fatty acids in PG has implicated to have an important role in the function of photosystem protein complexes for photosynthesis (Moon *et al.*, 1995; Somerville, 1995). Interestingly, our findings show that the relative levels of PG declined from wild-type to mutant by 283.2 to 230.2 nmol phosphorous/g tissue, respectively (approximately 18.7% decrease in the mutant) (Table 1). Furthermore, the polyunsaturated fatty acids, 18:2 and 18:3 of the PG and 18:3 of the MGDG and DGDG, were markedly decreased with 5.8-fold increase of 14:0 in the MGDG of the mutant compared to wild-type (Table 3). Thus, our observation of the changes mainly in PG and galactolipids suggests that the molecular environments of the photosystem on thylakoid membrane of the mutant may be altered and the changes might have affected the stability of the photosynthetic machinery and photosynthesis activity. This speculation should be tested systematically for photosystem activity and stability, and further photoinhibition of photosynthesis activity by comparing the rate of evolution of O_2 from thylakoid membrane of the amp 1-4 mutant.

Inositol phospholipids showed a generally increased trend in the mutant. PI, L-PI, PIP, and PIP_2 were increased 1.5, 3, 1.4, and 1.6 folds, respectively (Table 2). With regards the results of the fatty acid analysis shown in Table 4, PI did not show significant changes except for a slight decrease of 18:3 in the mutant. L-PI showed decreases of 16:0 and 18:3 and a 4-fold increase of 16:1. In the case of PIP, all the fatty acids showed a decreased pattern except for slight increases of 18:1 and 18:2. PIP_2 did not show any significant changes but 16:1 was increased 1.9-fold and 18:3 decreased 1.9-fold. Thus, the data from Tables 2 and 4 indicate inositol phospholipid metabolism is more actively functioning in the mutant as noticed by the increase of all the inositol phospholipids, especially the 3-fold increase of L-PI with 4-fold increase of 16:1 in the mutant. However, it remains to be elucidated whether the phospholipase-mediated polyphosphoinositides signal transduction mechanism also actively functions in the mutant.

In conclusion, our findings collectively provide correlative evidence and suggest that the consequence of alterations in the lipid composition of the amp 1-4 mutant evidenced by the increase of PE, the decrease of PG, the increase of inositol phospholipids, and the altered fatty acid composition is causally related to the phenotypic changes affecting the cellular and molecular morphology of its cell membranes. Moreover, the alterations might cause the dramatic difference in the metabolic activities that regulate or influence lipid composition and membrane function in the amp 1-4 mutant.

Acknowledgements This investigation was supported in part by funds from the Korea Science and Engineering Foundation (95K3-0401-01-03-1 and 96K3-0401-01-03-3 to PMBBRC, and 961-0507-057-2) to Y. P.

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